SCAN AS SPEC

EXAMINER'S INFORMAL AMDT

Amendments to the Specification:

Please replace the paragraph beginning at page 4, line 13 with the following rewritten paragraph:

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Fig. 1 shows a comparison of *L. delbrueckii* operator~~promoter~~ sequences (O1 and O2) as represented by SEQ ID NOs: 22-27. Arrows are for inverted repeats. The LL44 sequence is numbered according to Figure 1. Sequence of the second helix of *lacR* (repressor) is indicated.

Please replace the paragraph beginning at page 4, line 15 with the following rewritten paragraph:

Fig. 2 shows the organization of the promoter region of the *L. delbrueckii* LL44 and LB68 *lac operon* as represented by SEQ ID NOs: 30 and 31. Operators O1 and O2 are indicated by darkened boxes. The inverted repeats of the operators are represented by arrows. The sequence responsible for catabolite repression (CRE) is overdrawn by stars. The promoter sequence of LL44 is numbered according to Figure 1.~~;~~

Please replace the paragraph beginning at page 4, line 17 with the following rewritten paragraph:

Fig. 3 shows the organization of the promoter region of the *L. delbrueckii* N299 *lac operon* as represented by SEQ ID NOs: 32 and 33. Operators O1 and O2 are indicated by darkened boxes. The inverted repeats of the operators are represented by arrows. The sequence responsible for catabolite repression (CRE) is overdrawn by stars. The inverted repeat of ISL5 is boxed and shaded. The initiation of transcription is shown by an i (arrow head) (Leong-Morgenthaler et al., 1991).~~;~~

Please replace the paragraph beginning at page 4, line 19 with the following rewritten paragraph:

Fig. 4 shows the nucleotide and amino acid sequence of the *L. delbrueckii* subsp. *lactis* LL44 *lacR* gene as represented by nucleotides 1-1281 of SEQ ID NO:1 and SEQ ID NO:2, respectively. Start (121) and stop (1119) codons are boxed. Putative *lacR* RBS is underlined. The putative rho-indepedent terminator is underlined by convergent arrows. Stop codons of the beta-galactosidase (*lacZ*) and Asn t-RNA synthetase (*asnA*) genes are boxed. Insertion sequence of ISL*3* is represented by a large open arrow. Single base pair deletion (722) in the mutant LZL102 is shown by an arrow head, leading to a premature stop codon (758) underlined.~~;~~

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integer of ≥ 0; R denotes the gene coding for the ~~lacZ~~ lacR repressor protein as identified under SEQ ID No. 2 or a functional variant thereof; and Y is 0 or 1.

Consequently, in case y = 0 the gene coding for the lac repressor protein may be situated at any site of the bacteria's chromosome and may be subject to any suitable promoter region such that is e.g. expressed constitutively.

According to a preferred embodiment the DNA sequence is represented by the general formula p/o - $(A)_n$ - R or p/o - R - $(A)_n$, respectively, with p, A and R being as identified above. In this case, i.e. in the case of Y = 1, the construct is composed of three components, the promoter/operator region, the gene coding for the polypeptide of interest and the gene coding for the lac repressor protein (lacR), with the genes coding for the respective polypeptides (A, lacR) being arranged in either order. The lacR gene is such subject to its own promoter/operator. In addition, the gene coding for the lacR protein may be arranged such that its transcriptional direction is opposite to that of the gene A coding for the polypeptide of interest.

According to another preferred embodiment the gene coding for a polypeptide of interest is selected from group consisting of genes encoding enzymes, cell surface proteins or functional peptides, such as e.g. dextransucrase, glycosyltransferase, phytase, transglutaminase, peptidase, phenylalanine ammonia lyase, protease, cell surface antigens, bacteriocins, hormones, insulin.

The promoter region may also lack any catabolite responsive elements, so that no repression in the presence of a particular carbon source may be effected.

According to another preferred embodiment the DNA sequence is used to transform a microorganism, which is preferably a gram positive bacterium or more preferably a microorganism selected from the group consisting of lactic acid bacteria, such as Lactococcus, Lactobacillus, Streptococcus, Leuconostoc etc..

(CRE). This element is present in a variety of gene arrangements encoding carbon catabolite enzymes in different gram positive microorganisms and seems to be controlled by the trans acting factors CcpA (Catabolite control protein A) and Hpr. In the presence of glucose both of these proteins bind to the CRE sequence acting as a negative regulation of transcription.

The species L. delbrueckii contains mainly two subspecies, L. lactis and L. bulgaricus, which are defined by several physiological and genetic criteria. One of these criteria concerns the regulation of the lactose (lac) operon expression. In the subspecies lactis, the expression is induced by lactose, that is the lac genes are expressed only in the presence of this sugar. In the subspecies bulgaricus, which has been selected a long time ago for its ability to ferment milk for yogurt production, the control of the lac genes expression was lost probably due to the constant availability of lactose in milk and the genes are constitutively expressed in the presence or in the absence of lactose.

For the experiments leading to the present invention six representative strains have been chosen to study the regulation of genes via the lac operon. Five strains belong to the subspecies lactis and have been termed LL44, LB68, N62 and N141, which are inducible by the presence of lactose, and LB10, that has been classified as belonging to the subspecies lactis but with a constitutive expression of the lac operon. The sixth strain, N299, corresponding to ATCC 11842, was chosen as a typical bulgaricus strain, with a constitutive expression of the lac genes.

During the analysis of the lactobacillus strains the region flanking the lactose (lac) operon was isolated and the following regions could be characterized.

The lac A gene

During the study of the promoter region of the lac operon, an open reading frame (ORF) of 570 bp was discovered upstream the lacS gene in the same orientation. (see Figures 2 and 3) This gene was completely sequenced for the L. delbrueckii subsp. lactis strain LL44 ~~(SEQ ID. No 1)~~ and it encodes a polypeptide, lac A, of 190 amino acid residues ~~(SEQ ID. No. 2)~~. The presence of a

putative promoter and a potential ρ-independent-termination signal (stem-loop structure ~~position 823, SEQ ID NO 1~~) suggest that this gene does not belong to the lac operon.

The promoter region

The region comprised between the end of lacA and the beginning of lacS (lactose permease) was sequenced and showed several attributes specific for a promoter. Two palindromic sequences, called O1 and O2 (Fig. 1-3A), were discovered and may serve as operators for the binding of the lac repressor (LacR). The operators of the 6 strains studied (LL44, LB68, N62, N141, LB10 and N299) were sequenced. Several differences were found, which were partly due to the insertion of IS-elements in the O1 inverted repeats and partly due to small nucleotides changes in the sequence (Fig. 1). ISL7 is inserted in N141 exactly at the TGT motif of the 5' end of O1, but restoring the bases TG (Fig. 1). In N299, the L. delbrueckii subsp. bulgaricus strain, the insertion of ISL5 at the same position destroyed the TGT motif, which might explain the constitutive transcription of adjacent genes.

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In lactic acid bacteria, effective repression of transcription is deemed to involve the cooperative binding of LacR to two different operator sequences. In L. delbrueckii, a small operator, called O2, was found 4 bp downstream O1. The length of O2 showed considerable variation in length between the 6 strains analysed due to small nucleotide changes in the sequence (Fig. 1). However, no IS-element was found in this operator. The core of both inverted repeats is composed of the nucleotides TGTTTA (SEQ. ID. No. 3) and GTAACA (SEQ. ID. No. 4), except in LL44 where the final A was replaced by G in O2 (SEQ. ID. No. 5).

A sequence of 14 nucleotides homologous to the catabolite responsive element (CRE) (Weickert, supra), was discovered 40 nucleotides upstream the lacS start codon of all the L. delbrueckii strains studied (Fig. 2, Fig. 3, 1-14 of SEQ ID No. 8)). Catabolite repression is effective in many bacteria and acts at the level of transcription via negative transcriptional control. It involves a cis-acting element mediating repression of the genes under its control in the presence of glucose. The sequence of these elements is highly conserved among

different species and the one of L. delbrueckii subsp. lactis is highly similar to other elements.

The figures 2 and 3 present a view of the promoter region of the lac operon of 5 of the strains studied. They are considered as representative of the different possible lac operon organisations in the L. delbrueckii species. Four inducible lactis strains, LL44, LB68, N62 and N141 and one constitutive bulgaricus strain, N299 were represented. Strain LB10 was not shown, because its promoter region is identical to N62 except that ISL6 is not present in the lac promoter of this strain. The initiation of transcription in N299 (Leong-Morgenthaler et al., J. Bacteriol. 173 (1991), 1951-1957) falls in the middle of the CRE element, just after the three nucleotides change that were found in the CRE element of this strain (Figs. 2 and 3).

LL44 was chosen as a reference due to the absence of any IS-element in its lac operon region. In this strain, the lacA gene is followed by the two operators O1 and O2 and the CRE element upstream the lacS gene. In other strains, the presence of IS-elements changed the promoter sequence and particularly the O1 operator sequence.

The lac R gene

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Surprisingly, and in contrast to the gene arrangements of known lac operons, the gene coding for the repressor protein, lac R, was discovered downstream the lacZ gene in L. delbrueckii subsp. lactis LL44 and LB68, with the result that the repressor is under its own transcriptional control.

The region covering the lacR gene was PCR amplified using the following primers:

CGCCTGGTGATTCAGCC (SEQ ID No. 6)

AGCTTTACGGGGAAGTCGGG (SEQ ID No. 7)

which are located at the end of the ß-galactosidase (lacZ) gene (for SEQ ID No. 6) and in the Asn-tRNA synthetase (asnA) gene (for SEQ ID No. 7).

Sequencing of this region revealed the sequence identified under SEQ ID No. 1, which exhibits an open reading frame of 996 bp in the same orientation as lacZ. The lacR gene is preceded by a ribosome binding site (RBS) and followed by a putative ρ-independent-termination signal (stem-loop structure ; position 1149). The putative amino polypeptide derived therefrom is shown under SEQ ID No. 2 (332 amino acid residues).

Computer-assisted analysis of the L. delbrueckii lacR gene predicted the protein secondary structure of the beginning of the gene (positions 4 to 23) to be a helix-turn-helix motif, which represents the highest homology region with other repressors. This type of protein secondary structure is a common feature for DNA-binding proteins and binds to the operators located in the promoter region.

The lac operon

The organisation of the lac operon of different L. delbrueckii strains is represented in figure 5. The lac operon per se is preceded by the thiogalactosyl-transacetylase (lacA) gene, which is followed by a rho-independent termination signal. This gene is followed by the promoter region of the lac operon, which comprises the inverted repeats (operators) involved in the regulation of the gene expression and the CRE sequence. The lac operon is composed of three genes, the permease (lacS), the ß-galactosidase (lacZ) and the repressor (lacR). The lacS and lacZ genes are separated by 4 nucleotides, whereas lacR is distant from lacZ by 52 nucleotides.

Five IS-elements have been found in the promoter and the terminator region of the lac operon, which were called ISL3, ISL4, ISL5, ISL6 and ISL7. One of these Elements, ISL3, has been described in Germond et al, Mol. Gen. Genet. **248** (1995), 407-416.

The lactose permease (lacS), the ß-galactosidase (lacZ) and the repressor (lacR) constitute the lac operon per se. The three genes are linked together as a lacSZR operon in L. delbrueckii subsp. lactis without any promoter in between. This gene arrangement is not a common feature for regulated operons, as the lacR gene is normally not a part of the operon.

by Overlap Extension (gene SOEing) method (Horton R., Molecular Biotechnology 3 (1995), 93 – 99).

The promoter of lacI was first amplified from pET11c using the following primers

Pst I ATAAATCTGCAGTGGGTATGGTGGC	SEQ ID No. 16
GATCGTTGCCACATTCACCACC	SEQ ID No. 17

The primer SEQ ID No. 18 is composed of a sequence of the lacI promoter and of the L. delbrueckii 5' end of lacR. The complete lacR gene was then PCR amplified using the following primers

GGTGAATGTGGCAACGATCAG	SEQ ID No. 18
Pst I ATATTACTGCAGACAGAATGCAGCC	SEQ ID No. 19

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~~The sequence of primer SEQ ID No. 19 is the complement of SEQ ID No. 20.~~ Both PCRs were purified, mixed and reamplified with primers SEQ ID No. 16 and SEQ ID No. 19, linking the lacI promoter and the LL44 lacR gene. Additionally, the ATG start codon of the lacR gene was replaced by GTG as in lacI (Fig. 6). The whole construct was cloned in the PstI site of the low copy number plasmid pACYC177 (New England Biolabs) digested with PstI and resulted after transformation in plasmid pLL62 (Fig. 6). The presence of the modified lacR gene in pLL62 was confirmed by digestions and PCR amplifications.

This plasmid was cotransformed with pLL55, pLL57 and pLL58 in E coli resulting in strains LZL63, LZL64 and LZL65 (Table I) respectively. The resulting strains contained the different L. delbrueckii promoters together with the LL44 lacR gene. Regulation of the promoters was tested following the chloramphenicol acetyltransferase assay of Shaw, supra in the presence of lactose or glucose. The results showed that after 4 min incubation at 37°C, strains containing the different promoters were fully induced in YT medium or in a minimal medium based on M9-salts in the presence or in the absence of lacR as shown in the table below :